ALZHEIMER'S RELATED PROTEINS AND METHODS OF USE

CROSS REFERENCE TO RELATED APPLICATION

This application claims benefit of U.S. Provisional Application No. 60/070,948, filed January 9, 1998, the disclosure of which is hereby incorporated by reference herein.

FIELD OF THE INVENTION

The present invention relates generally to the field of neurological and physiological dysfunctions associated with Alzheimer's Disease. More particularly, the invention is concerned with the identification of proteins associated with Alzheimer's Disease, to methods of diagnosing Alzheimer's Disease and to methods of screening for candidate compounds which modulate the interaction of certain proteins, specifically *armadillo* repeat proteins, with presenilin proteins.

BACKGROUND OF THE INVENTION

Alzheimer's Disease (AD) is a degenerative disorder of the human central nervous system characterized by progressive memory impairment and cognitive and intellectual decline during mid to late adult life (*Katzman*, 1986, *N. Eng. J. Med.* 314:964-973). The disease is accompanied by a constellation of neuropathologic features principal amongst which are the presence of extracellular amyloid or senile plaques, and neurofibrillary tangles in neurons. The etiology of this disease is complex, although in some families it appears to be inherited as an autosomal dominant trait. Genetic studies have identified three genes associated with the development of AD, namely: (1) β-amyloid precursor protein (βAPP) (*Chartier-Harlin et al.*, 1991, *Nature* 353:844-846; *Goate et al.*, 1991, *Nature* 349:704-706; *Murrell et al.*, 1991, *Science* 254:97-99; *Karlinsky et al.*, 1992, *Neurology* 42:1445-1453; *Mullan et al.*, 1992, *Nature Genetics* 1:345-347);

(2) presenilin-1 (PS1) (*Sherrington et al.*, 1995, *Nature* 375:754-760); and (3) presenilin-2 (PS2) (*Rogaev et al.*, 1995, *Nature* 376:775-778; *Levy-Lehad et al.*, 1995, *Science* 269:970-973).

The presenilin genes (presenilin 1 - *PS1* and presenilin 2 - *PS2*) encode homologous polytopic transmembrane proteins that are expressed at low levels in intracellular membranes including the nuclear envelope, the endoplasmic reticulum, the Golgi apparatus and some as yet uncharacterised intracytoplasmic vesicles in many different cell types including neuronal and non-neuronal cells (*Sherrington et al.*, 1995; *Rogaev et al.*, 1995; *Levy-Lahad et al.*, 1995; *Doan et al.*, 1996, *Neuron* 17:1023-1030; *Walter et al.*, 1996, *Molec. Medicine* 2:673-691; *De Strooper et al.*, 1997, *J. Biol. Chem* 272:3590-3598; *Lehmann et al.*, 1997, *J.Biol.Chem.* 272:12047-12051; *Li et al.*, 1997, *Cell* 90:917-927). Structural studies predict that the presenilins contain between six and eight transmembrane (TM) domains organized such that the N-terminus, the C-terminus, and a large hydrophilic loop following the sixth TM domain are located in the cytoplasm or nucleoplasm, while the hydrophilic loop between TM1 and TM2 is located within the lumen of membranous intracellular organelles (*Doan et al.*, 1996; *De Strooper et al.*, 1997; *Lehmann et al.*, 1997).

Missense mutations in the PS1 and PS2 genes are associated with the inherited forms of early-onset AD (*Sherrington et al.*, 1995, *Nature* 375:754-760; *Rogaev, et al.*, 1995, *Nature* 376:775-778; *Levy-Lahad et al*, 1995, *Science* 269: 970-973). Several lines of evidence have also suggested roles in developmental, apoptotic signalling and in the regulation of proteolytic cleavage of the b-amyloid precursor protein (bAPP) (*Levitan et al.*, 1995, *Nature* 377:351-354; *Wong et al.*, 1997, *Nature* 387:288-292; *Shen et al.*, 1997, *Cell* 89:629-639; *Wolozin et al.*, 1996, *Science* 274:1710-1713; *De Strooper et al.*, 1998, *Nature* 391:387-390). Nevertheless, it remains unclear just how these putative functions are mediated, or how they relate to the abnormal metabolism of the βAPP associated with PS1 and PS2 mutations (*Martin et al.*, 1995, *NeuroReport*

7:217-220; *Scheuner et al.*, 1996, *Nature Med.* 2:864-870; *Citron et al.*, 1997, *Nature Med.* 3:67-72; *Duff et al.*, 1996, *Nature* 383:710-713; *Borchelt et al.*, 1996, *Neuron* 17:1005-1013).

The identification and cloning of normal as well as mutant PS1 and PS2 genes and gene products are described in detail in copending commonly assigned U.S. Application Serial Nos. 08/431,048, filed April 28, 1995; 08/496,841, filed June 28, 1995; 08/509,359, filed July 31, 1995; and 08/592,541, filed January 26, 1996, the disclosures of which are incorporated herein by reference.

There is speculation that onset of AD may be associated with aberrant interactions between mutant presenilin proteins and normal forms of PS-interacting proteins, and these changes may increase or decrease interactions present with normal PS1, or cause interaction with a mutation-specific PS-interacting protein. Such aberrant interactions also may result from normal presenilins binding to mutant forms of the PS-interacting proteins. Therefore, mutations in the PS-interacting proteins may also be causative of AD.

While the identification of normal and mutant forms of PS proteins has greatly facilitated development of diagnostics and therapeutics, a need exists for new methods and reagents to more accurately and effectively diagnose and treat AD.

SUMMARY OF THE INVENTION

Applicants have discovered that both PS1 and PS2 interact specifically with at least two members of the *armadillo* family of proteins (GT24/Neuronal Plakoglobin Related Armadillo Protein and β-catenin) that are expressed in both embryonic and post-natal tissues. Moreover, the domains of PS1 and PS2 that interact with these proteins have been identified. Applicants have also discovered that mutations in PS1 and PS2 affect the translocation of β-catenin into the nucleus of both native cells and cells transfected with a mutant PS gene. These discoveries provide the basis for materials and methods useful in the diagnosis and

treatment of AD.

One object of the invention is directed to a method for identifying substances that alter the interaction of a presenilin protein with a presenilin-binding protein, comprising:

(a) contacting at least the interacting domain of a presenilin protein to a presenilin-binding protein in the presence of a test substance, and

(b) measuring the interaction of the presenilin protein and the presenilin-binding protein. Preferably, the interacting domain is contained in or contains the sequence of amino acid residues from about 260 to about 409 of a mutant PS1 protein, more preferably the sequence of amino residues from about 372 to about 399, in which the amino acid positions correspond to the wild-type human PS1 sequence defined by SEQ ID NO:1. When PS2 is used, the sequence of amino acid residues from about 266 to about 390 are preferred, more preferably the sequence of amino residues from about 350 to about 380, in which the amino acid positions correspond to the wild-type human PS2 sequence defined by SEQ ID NO:2.

Substances identified that alter the interaction of a mutant presenilin protein with a normal presenilin-interacting protein, as well as the interaction of a normal presenilin protein with a mutant presenilin-interacting protein, are putative candidates for use in the diagnosis and treatment of AD.

Another object of the invention is to provide methods of identifying substances that modulate the nuclear translocation of an *armadillo* protein, comprising:

(a) providing a culture of cells that express the *armadillo* protein and a mutant presenilin protein, or a functional fragment thereof that binds said *armadillo* protein;

(b) contacting said culture with a test substance;

(c) inducing nuclear translocation of said *armadillo* protein in said cells; and

(d) measuring levels of nuclear *armadillo* protein as compared to a control as an indication of modulatory activity of said test substance. Alternatively, step (d) may comprise the step of measuring the effects of altered nuclear translocation such as: (i) alterations in transcription of downstream genes such as βAPP, γ-secretase, or by alteration in the activity of a transcription reporter assay such as the Tcf/Lef-luciferase assay; (ii) alterations in cellular responsiveness to signalling molecules (e.g., *Wnt*, EGF) which use armadillo proteins for intracellular signal transduction; or (iii) alterations in cell:cell adhesion (e.g., synapse formation) mediated by cytoplasmic armadillo proteins.

Armadillo proteins of the present invention include, but are not limited to, hNPRAP, p0071 and β-catenin. Cells may be native or recombinant (i.e., the mutant PS gene and/or the *armadillo* protein gene are/is transgenic to the cell).

It is another object of the invention to provide methods for screening for carriers of presenilin alleles associated with AD or related disorders, comprising:

(a) obtaining cells from an individual to be tested for Alzheimer's Disease or a related disorder;

(b) culturing said cells with a substance which induces nuclear translocation of an *armadillo* protein; and

(c) measuring levels of nuclear *armadillo* protein as compared to a control as an indication of the presence or absence of presenilin alleles associated with Alzheimer's Disease or a related disorder. Alternatively, step (c) may comprise measuring effects of altered nuclear translocation such as: (i) alterations in transcription of downstream genes such as βAPP, γ-secretase, or by alteration in the activity of a transcription reporter assay such as the Tcf/Lef-luciferase assay; (ii) alterations in cellular responsiveness to signalling molecules (e.g., *Wnt*, EGF) which use armadillo proteins for intracellular signal transduction; or (iii) alteration

in cell:cell adhesion (e.g., synapse formation) mediated by cytoplasmic armadillo proteins.

Inducement of nuclear translocation of the *armadillo* protein is preferably performed by activating the *Wnt/armadillo* signal transduction pathway of the cells. Most preferably, activation is with a lithium salt (e.g., lithium chloride) or with methods such as with recombinant *Wnt* proteins (or invertebrate homologues, e.g., Wingless proteins) applied exogenously to the medium or via transfection into the test cells.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the amino acid sequences of GT24/hNPRAP and p0071 and the location of the 10 *armadillo* repeats.

DETAILED DESCRIPTION OF THE INVENTION

Applicants have identified the presenilin domain i.e., the interacting domain, that interacts with PS-interacting proteins, such as *armadillo* repeat proteins hNPRAP, p0071 and β-catenin, as including or contained in the sequence of amino acid residues from about 260 to about 409 of PS1 or corresponding residues from about 260 to about 390 in PS2. More preferably, the interacting domain contains or is contained in amino acid residues from about 372 to about 399 of PS1 or corresponding residues from about 350 to about 380 in PS2. The amino acid sequences of wild-type human PS1 and PS2 are shown in SEQ ID NO:1 and SEQ ID NO:2, respectively.

Mutant PS1 and PS2 genes, and their corresponding amino acid sequences are described in Applicants' co-pending U.S. Application Serial No. 08/888,077, filed July 3, 1997, and incorporated herein by reference. Examples of PS1 mutants include I143T, M146L, L171P, F177S, A260V, C263R, P264L, P267S, E280A, E280G, A285V, L286V, Δ291-319, L322V, G384A, L392V, C410Y and I439V. The mutations are listed with reference to their amino acid positions in SEQ ID NO:1. Examples of PS2 mutants include N141I, M239V and I420T. These mutations are listed with reference to their amino acid positions in

SEQ ID NO:2. PS1 mutations A260V, C263R, P264L, P267S, E280A, E280G, A285V, L286V, Δ291-319, G384A, L392V, and C410Y all occur in or near the interacting domain of PS1 described herein. However, the methods of the present invention are not limited to mutant presenilins wherein the interacting domain is mutated relative to the wild-type protein. For example, Applicants have observed that mutations in PS1 (e.g., M146L) which are not in the interacting domain (loop) also affect β-catenin translocation. These mutations probably disturb the presenilin *armadillo* interactions by altering the function of a high MW complex which contains the presenilin and *armadillo* proteins (plus others), as described in *Yu et al.*, 1998, *J.Biol.Chem.* 273:16460-16475; *Levesque et al.*, 1999, *J. Neurochem.*, in press; and *Nishimura et al.*, 1999, *Nature Medicine*, in press. Moreover, a comparison of the hPS1 and hPS2 sequences reveals that these pathogenic mutations are in regions of the PS1 protein which are conserved in the PS2 protein. Therefore, corresponding mutations in corresponding regions of PS2 may also be expected to be pathogenic and are useful in the methods described herein.

Proteins that interact with the presenilins, i.e., PS-interacting proteins, include the S5a subunit of the 26S proteasome (GenBank Accession No. 451007), Rab11 (GenBank Accession Nos. X5670 and X53143), retinoid X receptor B, also known as nuclear receptor co-regulator or MHC (GenBank Accession Nos. M84820, X63522 and M87166) and GT24 (GenBank Accession No. U81004). These and other PS1 binding proteins such as *armadillo* proteins are described in Applicants' copending commonly assigned U.S. Application Serial No. 08/888,077, filed July 3, 1997, as well as U.S. Application Serial No. 08/592,541, filed January 26, 1996, the disclosures of which are incorporated herein by reference. The interaction between PS1 and β-catenin is reported by *Zhou et al.*, 1997, *Neuro. Report* (Fast Track) 8:1025-1029 and *Yu et al.*, 1998; *Levesque et al*, 1999; and *Nishimura et al.*, 1999. Mutant forms as well as wild-type presenilin-interacting proteins may be used in the methods described herein. By presenilin-interacting proteins it is meant full-length proteins or fragments that

contain the domain that interacts with the presenilin-interacting domain of a presenilin protein.

A first embodiment is directed to a method for identifying substances that alter the interaction of a presenilin protein with a presenilin-binding protein or which alter the functional consequences of the interaction. Candidate compounds which are shown to modulate these interactions may be produced in pharmaceutically useful quantities for use in the diagnosis and treatment of AD or related disorders. Candidate compounds include endogenous cellular components which interact with the presenilins *in vivo* and which, therefore, provide new targets for pharmaceutical and therapeutic interventions, as well as recombinant, synthetic and otherwise exogenous compounds which may have presenilin binding capacity and, therefore, may be candidates for pharmaceutical agents. Thus, in one procedure, cell lysates or tissue homogenates (e.g., human brain homogenates, lymphocyte lysates) may be screened for proteins or other compounds which bind to one of the normal or mutant presenilins. Alternatively, any of a variety of exogenous compounds, both naturally occurring and/or synthetic (e.g., libraries of small molecules or peptides), may be screened for presenilin binding capacity. In each of these embodiments, an assay is conducted to detect binding between a presenilin component containing at least the interacting domain of a presenilin protein described herein and some other moiety. Binding may be detected by indirect functional measures reflecting the functional consequences of the interaction (e.g., changes in intracellular Ca^{2+}, Na^{+}, K^{+}, or GTP/GDP ratio, changes in apoptosis or microtubule associated protein phosphorylation, changes in Aβ peptide production or changes in the expression of other downstream genes which can be monitored by differential display, 2D gel electrophoresis, differential hybridization, or SAGE methods) or by direct measures such as immunoprecipitation, the Biomolecular Interaction Assay (BIAcore) or alteration of protein gel electrophoresis. The preferred methods involve variations on the following techniques: (1) direct extraction by affinity chromatography; (2) co-

isolation of presenilin components and bound proteins or other compounds by immunoprecipitation; (3) BIAcore analysis; and (4) the yeast two-hybrid systems. Other procedures include methods which detect abnormal processing of PS1, PS2, APP, or proteins reacting with PS1, PS2, or APP (e.g., abnormal phosphorylation, glycosylation, glycation amidation or proteolytic cleavage) alterations in presenilin transcription, translation, and post-translational modification; alterations in the intracellular and extracellular trafficking of presenilin gene products; or abnormal intracellular localization of the presenilins.

A second embodiment is directed to a method for identifying substances that modulate the nuclear translocation of *armadillo* proteins, preferably β-catenin, hNPRAP (GT24) and p0071. Nuclear translocation assays can advantageously be used as a biological monitor of the effects of PS1 and PS2 mutations that cause AD. This interaction can be modulated by compounds which strengthen or weaken the interaction between PS1 and PS2 and *armadillo* repeat proteins. This interaction assay method finds use in both the diagnosis and treatment of AD.

Generally, paired cell lines (e.g., vertebrate cells, such as the ones used in the Examples described herein or invertebrate cells, such as *D. melanogaster* cells) may be used in the method. One set of cells (control), expresses wild-type PS1 and wild-type *armadillo* protein (e.g., hNPRAP, p0071, β-catenin). The second set of cells expresses mutant PS1 and wild-type *armadillo* protein (test). By the terms PS1 and PS2, it is meant full-length or functional fragments thereof (e.g., residues 260-409 of PS1) that bind a normal or mutant *armadillo* protein. By the term *armadillo* protein, it is meant full-length protein or functional fragments thereof (e.g., one or more *armadillo* repeats) that bind PS1 or PS2. For rapid *in vitro* assays, *armadillo* protein may be labelled with green-fluorescent protein (GFP) or blue fluorescence protein and transfected into the cells. PS1 or PS2 is preferably present in the form of a transgene, but cells in which PS1 and *armadillo* protein are endogenously expressed, such as patient fibroblasts or

cultured neurons from transgenic mouse brain, may be used. All vertebrate cells express β-catenin, but not all *armadillo* proteins are expressed in all cell types. Thus, cells may be transformed with a transgene encoding the *armadillo* protein of choice. *Armadillo* proteins and their corresponding nucleotide and amino acid sequences are known in the art. The sequence of hNPRAP (GT24) is described in U.S. Application Serial No. 08/888,077, filed July 3, 1997, and is incorporated herein by reference.

The cells are then exposed to a candidate substance to be tested plus an environment or agent that induces nuclear translocation of the *armadillo* protein. In a preferred embodiment, nuclear translocation is achieved by culturing the cells in the presence of a lithium salt such as lithium chloride, exogenous recombinant *Wnt/Wingless* protein, NGF, EGF, Aβ, kinase inhibitors (e.g., Herbanycin A, Genstein or Lavendustin A), or phosphatase inhibitors such as Na^+ Vanidate. These agents modulate the *Wnt/armadillo* signal transduction pathway in the cells. The last three named agents are less preferred than the first two agents (lithium and *Wnt/Wingless*) because they modulate phosphorylation of many proteins other than just β-catenin. In a most preferred embodiment, nuclear translocation is induced by contacting the culture with a lithium salt, preferably lithium chloride, or *Wnt/Wingless*.

Nuclear *armadillo* protein levels are preferably measured by direct visualization. Levels of nuclear *armadillo* protein in test cells versus controls cells are then compared to determine whether the test substance modulates the nuclear translocation of *armadillo* protein. In one preferred procedure, the cells are fixed and stained with anti-β-catenin antibodies (where β-catenin is the *armadillo* protein utilised). The amount of nuclear β-catenin is subsequently quantified by counting relative numbers of β-catenin (+) and β-catenin (-) nuclei, and/or by measuring the optic density of microscopic images of nuclei. In another procedure, cells containing GFP-tagged-β-catenin are directly visualised for translocation in living cells under UV light. Nuclear β-catenin levels may be quantified as above. An

advantage of this procedure is that it allows the same cell to be investigated using different conditions, for example using different quantities of the same or different test substances (drugs). In a further procedure, nuclei are isolated by known methods as described herein, and nuclear β-catenin levels are quantitatively measured by densitometry of signal intensity on Western blots probed with anti-β-catenin antibodies. In yet another procedure, nuclei from cells containing GFP-tagged β-catenin are isolated. Nuclear GFP-β-catenin is then quantified by direct measurement of GFP fluorescence signal intensity. In an alternative embodiment, the effects of altered nuclear translocation may be measured, e.g., measuring: (i) alterations in transcription of downstream genes such as βAPP, γ-secretase, or by alteration in the activity of a transcription reporter assay such as theTcf/Lef-luciferase assay; (ii) alterations in cellular responsiveness to signalling molecules (e.g., *Wnt*, EGF) which use armadillo proteins for intracellular signal transduction; or (iii) alterations in cell:cell adhesion (e.g., synapse formation) mediated by cytoplasmic armadillo proteins. Such procedures are well known to those skilled in the art, as described in *Korinek et al.*, 1998, *Mol.Cell.Biol.* 18:1248-56; *Morin et al.*, 1997, *Science*, 275:1787-90; *Korinek et al.*, 1997, *Science*, 275:1784-87; and *Molenaar et al.*, 1996, *Cell*, 86:391-92. In each of these procedures, the control may be cells that express the normal presenilin and *armadillo* protein genes.

The interacting domain of the presenilins can be used, together with presenilin binding proteins, or fragments thereof that bind to the interacting domain of PS1 or PS2, to screen for substances that alter (e.g., facilitate, interfere, inhibit, prevent) binding of PS1 and/or PS2 to presenilin binding proteins or alter the functional consequences of binding (i.e., nuclear translocation of *armadillo* proteins, changes in βAPP metabolism, transcription or translation, or changes in Aβ secretion). Such agents are candidates for use in the diagnosis or treatment of AD. Candidate substances may be selected from peptides, oligosaccharides, lipids, small molecules, compounds (drugs) or derivatives of any of the foregoing, or other molecules. Substances to be used in the screening methods of the invention may be

obtained e.g., from chemical or natural product libraries, including bacterial, fungal, plant and animal extracts. Substances can be tested in accordance with the invention for the ability to interfere with the binding of presenilin to a presenilin-binding protein. Such compounds may be found, for example, in natural product libraries, fermentation libraries (encompassing plants and microorganisms), combinatorial libraries, compound files, and synthetic compound libraries.

In yet another embodiment of the invention, a method is provided to screen for carriers of alleles associated with AD or related disorders, for diagnosis of victims of AD, and for screening and diagnosis of related presenilin and senile dementias, psychiatric diseases such as schizophrenia and depression, and neurological disease such as stroke and cerebral hemorrhage, associated with mutations in the PS1 or PS2 genes or presenilin binding proteins. Any eukaryotic cell may be used in the method. Control cells may be cells from a normal individual, i.e., absense of alleles associated with AD or related disorders. In preferred embodiments, fibroblasts, leukocytes or neuronal cells are used. The *armadillo* protein assayed depends upon the type of cells obtained from the subject. Induction of nuclear translocation and visualization of the *armadillo* protein are conducted as described above. In an alternative embodiment, the effects of altered nuclear translocation may be measured in the cells obtained from the subject, e.g., measuring: (i) alterations in transcription of downstream genes such as βAPP, γ-secretase, or by alteration in the activity of a transcription reporter assay such as the Tcf/Lef-luciferase assay; (ii) alterations in cellular responsiveness to signalling molecules (e.g., *Wnt*, EGF) which use armadillo proteins for intracellular signal transduction; or (iii) alterations in cell:cell adhesion (e.g., synapse formation) mediated by cytoplasmic armadillo proteins. Such procedures are well known to those skilled in the art, as described in *Korinek et al.*, 1998; *Morin et al.*, 1997; *Korinek et al.*, 1997; and *Molenaar et al.*, 1996.

In general, modifications of the sequences encoding the polypeptides described herein may be readily accomplished by standard techniques such as

chemical syntheses and site-directed mutagenesis. See *Gillman et al.*, 1979, *Gene* 8:81-97; *Roberts et al.*, 1987, *Nature* 328:731-734; and *Innis* (ed.), 1990, *PCR Protocols: A Guide to Methods and Applications*, Academic Press, New York. Most modifications are evaluated by routine screening via an assay designed to select for the desired property.

These and other aspects of the invention are described in more detail by reference to the following examples.

EXAMPLE 1

To identify cDNAs encoding PS1-binding proteins, a yeast two hybrid library containing human adult brain cDNAs was screened using three overlapping "bait" cDNA sequences derived from the $PS1_{266-409}$ cytoplasmic loop. These bait sequences were: 1) a wild type PS1 TM6-TM7 loop ($PS1_{266-409}$); 2) a PS1 Exon 10 splice mutant, which construct corresponds to the FAD-linked Δ290-319 mutation ($PS1_{266-289/320-409}$); and 3) a construct corresponding to the physiologic 18 kDa C-terminal cleavage fragment ($PS1_{290-467}$) which, in a six transmembrane (TM) model of PS1 topology, would be entirely cytoplasmic (*Lehmann et al.*, 1997, *J.Biol.Chem.* 272:12047-12051) but in an eight TM model would contain two additional TM domains (*Doan et al.*, 1996, *Neuron* 17:1023-1030).

The bait sequences were ligated into pAS2-1 (Clontech), were shown to be free of autonomous β-gal activation, and were independently co-transformed into Y190 *S. cerevisiae* together with a human brain cDNA library in pACT2 vector (Clontech). Transformants were selected for His^+, $\beta\text{-gal}^+$ activity, and the "trapped" candidate PS1 interacting cDNAs were isolated, sequenced, and analysed by BLASTN database searches. Negative control cDNAs included human Lamin C. Quantitative β-gal assays were performed according to the Matchmaker II protocol (Clontech).

Six of the 42 His^+, βgal^+ clones trapped by the wild type $PS1_{266-409}$ bait, one of ten clones trapped by the mutant $PS1_{260-289/320-409}$ bait, and one of six

clones trapped by the C-terminal $PS1_{290-467}$ bait represented overlapping clones derived from the same transcript, termed GT24. (GenBank Accession No. U81004.)

Nucleotides 2920-2997 of the GT24 cDNA overlap the anonymous microsatellite locus D5S478, therefore placing the GT24 gene on chromosome 5p15 near the Cri-du-Chat deletion locus, a syndrome associated with congenital malformation and gross mental retardation. This raises the distinct possibility that mutations or deletions in GT24 have a role in Cri-du-Chat syndrome. The GT24 sequence thus finds use in the diagnosis and therapy of Cri-du-Chat syndrome. On Northern blots, the GT24 gene is expressed as a range of transcripts of 3.9 to 5.0 kb in several regions of adult human brain, but is expressed at only very low levels in most non-neurologic tissues. The open reading frame (ORF) of the GT24 consensus cDNA encodes a protein of 1084 residues with a unique N-terminus, but with homology to proteins with *armadillo (arm)* repeat motifs at its C-terminus.

Searches of public nucleotide sequence databases also uncovered a murine orthologue of GT24, termed Neural Plakophilin Related Armadillo Protein (NPRAP), a protein of unknown function which contains approximately 147 additional amino acids at the N-terminus (*Paffenholz et al.*, 1997, *Differentiation* 61:293-304). It is unclear whether this difference reflects a true difference between the human and murine orthologues, or an artefact of cloning.

In addition to the GT24 clone (now known as human Neural Plakophilin Related Armadillo Protein (hNPRAP), one further His^+, βgal^+ yeast-two-hybrid clone (psl1y2h-25) was found to encode another peptide with C-terminal *arm* repeats. Clone psl1y2h-25 corresponds to a cDNA sequence deposited in GenBank as human protein p0071 (Accession Nos. U81005 and P18824). The ORF of psl1y2h-25/p0071 has 47% overall amino acid sequence identity with hNPRAP, and 70% identity to the *arm* repeats at residues 390-906 of hNPRAP.

The amino acid sequence alignment of hNPRAP (U81004)(bottom line) and ps1ly2h-25/p0071 (U81005 and P18824)(top line)(solid vertical lines = identity; dotted vertical lines = similarity) is shown in Figure 1. Since the high GC content of the 5' end sequences (87%) resulted in the recovery only of truncated cDNA clones, residues 1-44 of hNPRAP were derived from genomic DNA sequences. There are 10 putative *armadillo* repeats in hNPRAP which were identified using the *arm* consensus sequence (DKNDEKVVTCAAGTLHNLSVHNQNNKMIVRASGG) (SEQ ID NO:3) from PRODOM protein domain entry 138 (*Sonnhammer et al.*, 1994, *Protein Sci.* 3:482-492). These residues also show strong homology to *arm* repeats in other proteins such as P120cas (Z17804: 32-56% identity, $p=1.2 \times 10^{-133}$), human β-catenin (P35222:28-47% identity, $p=2.6 \times 10^{-4}$), and *D. melanogaster armadillo* (P18824: 26-43% identity, $p=1.9 \times 10^{-4}$).

Examples 2-5 illustrate both the specificity of the PS1:hNPRAP yeast two hybrid interaction and its occurrence under physiological conditions in mammalian cells.

EXAMPLE 2

Reciprocal immunoprecipitation (IP) experiments confirm that PS1 and hNPRAP specifically interact *in vitro* and that the hNPRAP:PS1 interaction requires the *arm* repeat of hNPRAP. Also shown is that hNPRAP interacts with PS2.

cDNAs encoding wild type PS1 (wtPS1) holoprotein, and various fragments of hNPRAP tagged at the C-terminus with a *myc*-tag (EQKLISEEDLN) (SEQ ID NO:4) and/or tagged at the N-terminus with a His-tag, green fluorescent protein (e.g. myc-hNPRAP$_{528-1084}$), or Xpress-epitope tag (Invitrogen) were either transiently expressed (hNPRAP) or stably expressed (PS1) from the pcDNA3 vector (Invitrogen) or from the pEGFP-C1 vector (Clontech, Palo Alto, CA) in HEK293 cells. Endogenous β-catenin, α-catenin, γ-catenin, and calnexin were used.

Co-immunoprecipitation and glycerol velocity gradient fractionatioin were performed as previously described in *Yu et al.*, 1998, *J.Biol.Chem.* 273:16470-16475. Immunoprecipitations were performed on cell lysates 48 hours after transient transfections. Total proteins (2 mg/ml) were extracted from cultured cells or brain tissue using a lysis buffer (0.2% NP-40, 0.5% Triton X-100, 50 mM Tris-HCl [pH7.6], 150 mM NaCl, 2 mM EDTA, 2 mM PMSF, 2 mg/ml each of aprotinin, leupeptin, pepstatin). 1.0 mg proteins were incubated overnight with appropriate antibodies (anti-NPRAP [Y120], anti-PS1 antibody to the), C-terminus or to the TM6-TM7 $PS1_{260-409}$ cytoplasmic loop antibodies 520, 1143, 3027 (*Walter et al.*, 1996, *Molec. Medicine* 2:673-691), to c-myc [9E10.2], β-catenin, α-catenin, γ-catenin (Transduction Laboratories, Lexington, KY), calnexin (Stressgen, Vancouver, BC) or pre-immune serum as described in *Harlow et al.*, 1988, Antibodies: A laboratory manual. New York, Cold Spring Harbor Laboratory Press. Protein A Sepharose was subsequently added to the antibody-antigen complex and incubated for 2 hours at 4^0C. The beads were washed four times with a washing buffer (0.2% NP-40, 50 mM Tris-HCl[pH7.6], 150 mM NaCl, 2 mM EDTA). IP products were resolved by SDS-PAGE and investigated for the co-immunoprecipitated partners with the corresponding antibody and detection with ECL (Amersham). 5% of the starting detergent lysate (50 mg) was loaded onto the cell extract lane. In experiments using cross-linking, cells were pretreated with 1 mM dithiobis-succinimidyl-proprionate (DSP) for 20 min. on ice.

For glycerol velocity gradient fractionation, brain tissue (1.0 g) was homogenized in a total volume of 5.0 ml of 25 mM Hepes, pH 7.2 at 4°C with the protease inhibitors described above, and as described in *Yu et al.*, 1998. After spinning at 1000 x g for 15 minutes to remove cell debris and nuclei, the resulting supernatant was centrifuged at 100,000 x g for 60 minutes to collect cell membranes. The membranes were then washed for 45 minutes with a KCl buffer (1 M KCl, 25 mM Hepes, pH 7.2, and protease inhibitors) and centrifuged again at 100,000 x g for 60 min. Cell membranes were then lysed with 1.0 % Digitonin in 25 mM Hepes, pH

7.2, 100 mM KCl and protease inhibitors. 0.5 ml of membrane extracts (2 mg/ml) was applied to the top of a 11.5 ml 10-40% (w/v) linear glycerol gradient containing 25 mM Hepes, pH 7.2, 150 mM NaCl, 0.25% Digitonin. Gradients were centrifuged for 15 hours at 35,000 rpm and 4 °C using an SW41 rotor and collected by upward displacement into 1.0 ml fractions using an Isco model 640 density gradient fractionator.

Myc-tagged hNPRAP$_{528\text{-}1084}$ was detected in whole lysates of *myc*-hNPRAP transfected control cells; in IP products from double-transfected HEK293 cells using the anti-PS1 N-terminal antibody 14.3 with prior DSP cross-linking; using anti-PS1$_{260\text{-}409}$ loop antibody with cross-linking or without prior cross-linking. No *myc*-hNPRAP was detected in cells transfected with hNPRAP only and immunoprecipitated with anti-PS1 loop antibody, or in any cell precipitated with pre-immune (PI) serum or with beads but no antibody.

PS1 holoprotein was detected using anti-PS1 N-terminal antibody 14.3 in Western blots of whole lysates from a PS1-transfected control cells and in the anti-*myc* IP products of a double-transfected cell with no cross-linking.

No PS1 was detected in anti-*myc* IP products from cells transfected with *myc*-hNPRAP only, PS1 only, or precipitated with an irrelevant antibody.

PS1 holoprotein and C-terminal PS1 fragments were detected using anti-PS1 N-terminal and anti-loop antibodies (mixed) in Western blots of lysates from HEK293 cells stably expressing PS1 and transiently transfected with hNPRAP cDNA. PS1 holoprotein and C-terminal PS1 but not N-terminal PS1 were co-precipitated with *his*-tagged hNPRAP (residues 45-1085) on Ni-Agarose columns. An irrelevant *his*-tagged control protein (His-LacZ) did not co-precipitate any PS1. PS1 alone did not precipitate on the Ni-Agarose column.

Western blots using anti-*myc* antibody (9E10.2) detected *myc*-tagged hNPRAP in immunoprecipitates of HEK293 cells stably expressing PS1 and transiently transfected with *myc*-hNPRAP immunoprecipitation with anti-PS1 loop antibody 1143 and in immunoprecipitates of HEK293 cells stably expressing PS2

and transiently expressing *myc*-hNPRAP immunoprecipitation with anti-PS2 antibody 972. No *myc*-tagged hNPRAP was detected in IP products from PS2-only transfected cells or cells immunoprecipitated with pre-immune serum.

In cells expressing PS1 and arm^+-hNPRAP, anti-*myc* antibodies (9E10.2) detected *myc*-tagged-arm^+ hNPRAP peptide (GT24 residues 320-1085) in whole lysates and in anti-PS1 immunoprecipitates (anti-PS1 loop antibody 1143), but not in immunoprecipitates using pre-immune serum. The *myc*-tagged N-terminal arm^--hNPRAP peptide (residues 45-413) was detected in whole lysates of cells doubly transfected with PS1 and arm^--hNPRAP, but not in immunoprecipitates using either anti-PS1 antibodies or pre-immune serum.

This example shows: (1) PS1 and hNPRAP can be reciprocally co-immunoprecipitated from lysates of cells doubly transfected with full-length human PS1 and *myc*-tagged hNPRAP (*myc*-$hNPRAP_{528-1084}$). However, co-immunoprecipitation did not occur in single transfected cells or from lysates of cells immunoprecipitated with pre-immune serum, with antibodies to unrelated proteins, or with Protein A Sepharose but no antibody; (2) a non-specific interaction between PS1 and hNPRAP can be excluded because neither protein co-precipitated with other irrelevant cellular proteins such as transfected His-LacZ or with other endoplasmic reticulum proteins such as endogenous calnexin (See Example 5); (3) the co-precipitation requires that the hNPRAP construct contain the C-terminal *arm* repeats (hNPRAP-arm^+). Co-immunoprecipitation does not occur when the hNPRAP transcript encodes only the unique arm^- N-terminus of hNPRAP. Conversely, the *arm*-binding domain of PS1 must include residues Thr_{320} to Ala_{409} because: 1) residues 320-409 are the only residues common to all three PS1 yeast two hybrid "bait" constructs; and 2) hNPRAP co-precipitates PS1 holoprotein and PS1-CTF, but does not co-precipitate PS1-NTF. Within the $PS1_{320-409}$ domain, residues 372-399 contain a single *arm*-motif (20% identity to *arm* consensus sequence) which is highly conserved in PS2 (91% identity) (*Rogaev et al.*, 1995, *Nature* 376:775-778), and in the invertebrate homologues (82%

identity) (*Levitan et al.*, 1995, *Nature* 377:351-354; *Boulianne et al.*, 1997, *NeuroReport* 8:1025-1029).

EXAMPLE 3

To exclude the remote possibility that both the yeast-two-hybrid and the co-immunoprecipitation studies in double transfected cells were artifacts arising from over-expression, both co-immunoprecipitation and glycerol velocity gradient analyses were used to investigate the PS1:NPRAP interaction *in vivo* in the mammalian central nervous system (CNS). A rabbit polyclonal antibody (Y120) directed to residues 156-170 of human NPRAP (which are predicted to be unique to NPRAP and do not cross react with other *arm* proteins) was generated. The specificity of this antibody was confirmed by showing that it specifically recognized *myc*-tagged NPRAP in transiently transfected HEK293 cells, that the Y120 immunoreactive band could be abolished by pre-absorption of the antibody with the cognate peptide, and that the Y120 immunoreactive bands were also detected by an anti-myc monoclonal antibody. Both the anti-*myc* and the anti-hNPRAP antibodies detected an abundant protein of ~130 kDa (corresponding to full-length *myc*-tagged NPRAP), and an ~75 kDa species which likely represents a C-terminal proteolytic derivative. The hNPRAP antibody also detected a ~120 kDa species which is not detected by the *myc*-antibody, and which is likely an N-terminal proteolytic derivative lacking the *myc* epitope tag. However, both of these lower molecular weight species likely represent aberrantly processed derivatives peculiar to overexpression in HEK293 cells because, in contrast to the full length NPRAP species, they are not detectable in human brain homogenates. To confirm the PS1:NPRAP interaction, anti-hNPRAP antibody was used to investigate anti-PS1 immunoprecipitates from human post-mortem neocortex, and to show that NPRAP is strongly detectable in these anti-PS1 immunoprecipitates. Glycerol velocity gradient analysis of lysates of murine neocortex revealed that NPRAP co-fractionates with the high molecular weight PS1-NTF and PS1-CTF containing complexes (~250 kDa).

EXAMPLE 4

In situ hybridizations in post-natal brain tissue were performed on 18 μm sections from approximately 4 month old murine brain (*Moser et al.*, 1991, *Neuron* 14:509-517). Digoxigenin-labelled antisense or sense strand cRNA probes were generated from the unique sequence at 788-1085 bp of hNPRAP cDNA (Boehinger Mannheim). Sections were hybridized with 100 μL of either antisense or sense probe at a final concentration of 200 ng/ml in 50% formamide, 3 x SSC, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 1% BSA, 500 μg/ml ssDNA, 500 μg/ml tRNA, 10 mM DTT, 10% dextran sulphate at 45°C overnight in a humidified chamber, and then processed as described by *Takeichi et al.*, 1995, *Curr. Opin. Cell Biol.* 7:619-627. Day E8.5, E10.5, E11.5 and E13.5 embryos ("E" = embryonic post-conception) were fixed in 4% paraformaldehyde, 30-40% sucrose, embedded in OCT and sectioned at 12 μm. Sections were processed as above except for the addition of digestion with 0.01% Proteinase K at 37°C for 20 min prior to the TEA acetic anhydride step.

In situ hybridization of digoxigenin-labelled anti-sense strand hNPRAP cRNA probes in 4 month post-natal mouse brain showed specific intense staining of neurons in hippocampus, dentate, scattered neocortical, cerebellar Purkinje and granule cells. There was prominent expression in CD1 mouse dorsal root ganglia and neural tube at day E13.5. Sense strand probes produced only minor background signals.

The *in situ* hybridization studies indicated that the transcriptional pattern of PS1 and NPRAP overlap in the brain of 4 month old mice and in murine embryos. In adult mouse brain both genes are expressed at high levels in dentate and hippocampal neurons, in scattered neocortical neurons, and in cerebellar Purkinje cells in adult mouse brain. (*Lee et al.*, 1996, *J. Neurosci.* 16:7513-7525; *Levesque et al.*, manuscript in prep; *Paffenholz et al.*, 1997, *Differentiation* 61:293-304). Similarly, there is overlap of the embryonic patterns of regional expression of PS1 and NPRAP. Both genes are prominently transcribed for instance in the

neural tube and dorsal root ganglia at day E10.5. Confocal laser micrographs of disassociated MDCK cells transiently expressing PS1 (PS1 antibody 14.3, FITC conjugated secondary, green color) and His-tagged-hNPRAP (using GT24 cDNA in pcDNA3.1 vector, Invitrogen) (anti-Express antibody, Rhodamine conjugated secondary, red color) showed colocalization of PS1 and hNPRAP. Experiments using *arm* constructs show diffuse cytoplasmic localization.

In MDCK cells forming intercellular contacts, Green Fluorescent Protein-tagged hNPRAP (pEGFP-C1 vector, Clontech, Palo Alto, CA) is predominantly localized near the cell membrane. Experiments using only *myc*-tagged hNPRAP showed similar results. These immunocytochemical studies in doubly-transfected CHO and MDCK cells revealed that transfected epitope tagged hNPRAP has a variable intracellular distribution. In disassociated cells, hNPRAP has a predominantly perinuclear cytoplasmic distribution contiguous with that of PS1. In contrast, in confluent cells with abundant cell:cell contacts, hNPRAP is predominantly located beneath the cell membrane close to inter-cellular contact zones.

The fact that only PS1 residues 320-409 are contained in all three PS1 yeast two hybrid "bait" constructs, the fact that the smallest hNPRAP "trapped" clone would be predicted to encode only the C-terminal *arm* repeat (residues 863-1084), and the results of the immunoprecipitation experiments cumulatively support that the PS1:hNPRAP interaction occurs between the C-terminal *arm* repeats in hNPRAP and residues Thr_{320} to Ala_{409} in PS1. Residues 372-399 of PS1 contain a single *arm*-motif (20% identity to *arm* consensus sequence) that is highly conserved not only in PS2 (91% identity) (*Rogaev et al.*, 1995, *Nature* 376:775-778), but also in the invertebrate homologues (82% identity) (*Levitan et al.*, 1995, *Nature* 377:351-354; *Boulianne et al.*, 1997, *NeuroReport* 8:1025-1029). As would be predicted, co-immunoprecipitation experiments in double transfected HEK293 cells reveal that PS2 also interacts with hNPRAP.

EXAMPLE 5

The validity of the PS1:hNPRAP interaction and the hypothesis that it arises from an interaction involving residues 372-399 of PS1 were confirmed by *in vitro* affinity chromatography experiments.

A His-tagged PS1 loop fragment corresponding to residues 266-409 was covalently linked to an Affi-Gel resin (BioRad) and then incubated with whole lysates of HEK293 cells stably expressing *myc*-tagged hNPRAP. The resulting complexes were washed repeatedly and the specifically interacting proteins were eluted in 1% SDS and examined by Western blotting using anti-*myc* antibodies. *In vitro* affinity chromatography suggests that the PS1:hNPRAP interaction involves the *arm*-repeat of hNPRAP and residues 372-399 of PS1. *myc*-tagged arm^+-hNPRAP (GT24/arm+) can be detected on Western blots of eluates from the affinity column containing immobilized PS1 cytoplasmic loop residues 266-409. The presence of large quantities of *myc*-tagged hNPRAP in these eluates clearly demonstrates a high affinity for the PS1 loop. Binding is absent with resin alone, and greatly diminished by pre-incubation with recombinant $PS1_{372-399}$ peptide. *myc*-tagged arm^--hNPRAP (GT24/arm-) was not detected in the column eluate, but was present in the column flow through. The specificity of this interaction is supported by the following observations. First, *myc*-hNPRAP does not bind non-specifically to the blocked resin alone. Second, *myc*-tagged hNPRAP lacking the *arm* repeats (hNPRAP residues 43-413) does not bind to the immobilized PS1 loop, but appears in the in the column flow-through. Third, other *myc*-tagged cytoplasmic proteins (e.g. *myc*-tagged anti-secretory factor) do not bind to the immobilized PS1 loop. Finally, binding of hNPRAP to the immobilized PS1 loop domain can be competitively inhibited by pre-incubation of the hNPRAP-containing cell lysates with a synthetic peptide corresponding to the PS1 *arm*-like sequence at PS1 residues 372-399. The hNPRAP:PS1 interaction, however, was not affected by pre-incubation with a control peptide corresponding to the TM1-2 loop of PS1 (residues 100-133).

EXAMPLE 6

The results described in Examples 2-5 support the notion that there is a specific interaction between PS1 and hNPRAP. Since, however, the yeast-two-hybrid studies also detected an interaction between PS1 and a closely-related *arm* protein PS1ly28-25/p0071, a study was conducted to determine whether PS1 might interact promiscuously with several members of the *arm*-repeat protein family.

Western blots of anti-PS1 immunoprecipitates from PS1 transfected HEK293 cells were examined for the presence of other *arm* proteins expressed endogenously in HEK293 cells. Endogenous β-catenin, but not endogenous α-catenin, γ-catenin, or calnexin were detected in Western blots of immunoprecipitation products from HEK293 cells stably transfected with PS1, using either antibodies to PS1 cytoplasmic loop or to the PS1 N-terminus. These studies reveal that the anti-PS1 immunoprecipitates contain endogenous β-catenin but not α-catenin or γ-catenin, and suggest that PS1 selectively interacts only with a subset of *armadillo* proteins. Endogenous β-catenin was also found to co-precipitate with transfected PS2 following immunopreciptiation of HEK293 cell lysates with anti-PS2 antibodies, but not with pre-immune serum.

EXAMPLE 7

In view of the effect of null mutations on developmental signalling pathways in *C. elegans* and mice (*Levitan et al.*, 1995, *Nature*, 377:351-354; *Wong et al.*, 1997, *Nature* 387:288-292; *Shen et al.*, 1997, *Cell* 89:629-639), the effect of mutations in PS1 and PS2 on the nuclear translocation of endogenous β-catenin following activation of the *Wnt/armadillo* signal transduction pathway by lithium induced inhibition of glycogen-synthetase-kinase-3β (*Stambolic et al.*, 1996, *Curr. Biol.* 6:1664-1668) was examined to determine whether the PS:*arm* interactions had a functional role.

Nuclear β-catenin was quantitatively assessed either by immunocytochemistry in native fibroblasts or by Western blotting of nuclear fractions from transfected HEK293 cells. Native fibroblasts obtained by skin

biopsy from normal subjects or subjects with PS1 or PS2 mutations were plated at low density. After approximately 65 hours the medium was replaced with media containing 20 mM lithium chloride for 3 hours. The cells were then fixed for 10 min. in 2% paraformaldehyde, incubated with 5% FBS for 30 min., stained with mouse monoclonal anti-β-catenin antibodies (1:500, Transduction Labs) at 4°C overnight, and counterstained with Hoechst 33342 dye (Molecular Probes) to label nuclei. β-catenin positive and negative nuclei were then directly counted in approximately 400 cells from different fields.

HEK293 cell lines stably transfected with wild type PS1 (wt2 and sw/wt6), Leu392Val-mutant PS1 (VL25 and VL31), wild type PS2 (sw2-9), Asn141Ile mutant PS2 (sw2-VG1) (kindly provided by Dr. D. Selkoe), and Δ290-319 mutant PS1 were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, and were treated with lithium chloride at 5 mM final concentration for 3 hours (*Stambolic et al.*, 1996, *Curr. Biol.* 6:1664-1668). Nuclei were collected from lithium treated and control cells as previously described in *Dignam et al.*, 1983, *Nucl. Acid Res.* 11:1475-1489). Cells were washed once in ice-cold PBS, resuspended in hypotonic lysis buffer (10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.05% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10μg/ml aprotinin, 5μg/ml leupeptin), and incubated for 30 minutes on ice. The nuclei were pelleted by microcentrifugation at 3,500 rpm for 2 minutes at 4°C, and cytoplasmic fractions were collected from the supernatants. Cytoplasmic soluble β-catenin fractions were incubated with Concavalin A-Sepharose beads (Pharmacia Biotech) as previously described (*Miller et al.*, 1997, *J. Cell Biol.* 139:229-243). Nuclear fractions were extracted by resuspending the nuclei in a high-salt buffer (20 mM Hepes, 400 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, 5 μg/ml leupeptin) and then incubated with Concavalin A-Sepharose beads to remove contaminating cadherin-bound β-catenin. 5 μg protein of each fraction was separated on 10% SDS-PAGE, and blots were probed with mouse monoclonal

antibody against β-catenin (25 ng/ml, Transduction Lab.). Signals were detected by ECL (Amersham) and were quantified from the autoradiographs by the NIH Image software package.

To assess nuclear translocation of NFκB, the same cells were incubated with medium alone or medium supplement with 50 ng/ml of Tumour Necrosis Factor-α (PeproTech Inc.) for 30 minutes, and then processed as above. Nuclear NFκB was quantified on Western blots of nuclear preparations using rabbit polyclonal antibody to p65 subunit (Santa Cruz Biotechnology, Santa Cruz, CA).

Endogenous β-catenin in mock treated native human fibroblasts was diffused throughout the cell, or predominantly cytoplasmic. After lithium chloride treatment of wild type fibroblasts, β-catenin is strongly localized in nuclei. In heterozygous PS1 mutant fibroblasts (e.g., Ala260Val PS1, Leu286Val PS1 and Met146Leu) lithium induces very little nuclear translocation. Western blot analyses of nuclear preparations showed progressive accumulation of endogenous β-catenin at 0, 30, and 60 minutes after lithium chloride incubation of HEK293 cells transfected with wild type PS1 (wtPS1) or PS2 (wtPS2) but not mutant PS1 (L392V) or mutant PS2 (N141I).

Treatment of the same cells with TNFα induced increased translocation of NFκB, but there were no differences between non-transfected, wild type or mutant PS1, or mutant PS2.

To investigate β-catenin ubiquitin:proteasome-mediated degradation pathways, HEK293 cells were incubated in medium containing 25 mM ALLN (N-acetyl-Leu-Leu-Norleucinal, Sigma) or ALLN plus 5 mM LiCl for 0, 1, 3, or 6 hours. Cells were harvested, lysates were prepared as previously described in *Yu et al.*, 1998, *J.Biol.Chem.* 273:16470-16475, and 10 mg of protein were subjected to SDS-PAGE and Western blotting followed by immunodetection using ECL and a mouse monoclonal anti-β-catenin antibody (Transduction Labs) as previously described in *Aberle et al.*, 1997, *EMBO J.* 16:3797-3804. Equivalent amounts of ubiquitinated and non-ubiquitinated β-catenin were detected in HEK293 cells

expressing wild type or mutant PS1 following treatment with a proteasome inhibitor ALLN alone or ALLN with LiCl.

Cleared lysates of HEK293 cells stably expressing mutant or wild type PS1 were immunoprecipitated with a rabbit polyclonal antibody to the $PS1_{260-409}$ loop (1143), the immunoprecipitates were separated by SDS-PAGE, blotted and probed with monoclonal anti-b-catenin antibodies as described by *Yu et al.*, 1998. Equivalent amounts of endogenous b-catenin were co-immunoprecipitated from HEK293 cells with transfected wild type PS1 or mutant PS1. The transfected HEK293 cells contained equivalent amounts of immunoprecipitable PS1 (wt, Leu392Val or D290-319).

This example shows that mutations in PS1/PS2 modulate the translocation of β-catenin into the nucleus. There were no differences in basal levels of nuclear β-catenin between mutant or wild type native fibroblasts, or between untransfected HEK293 cells and HEK293 cells stably transfected with either wild type or mutant PS1 or PS2 cDNAs. However, upon stimulation of the *Wnt/armadillo* signal transduction pathway by incubation in medium containing 20 mM lithium chloride for three hours, normal fibroblasts showed pronounced translocation of β-catenin into the nucleus (nuclear β-catenin in 33/41 cells). In contrast, nuclear translocation of β-catenin was significantly reduced in fibroblasts from heterozygous carriers of the Met146Leu (87/349), His163Tyr (176/383), Ala260Val (88/337) and Leu286Val (69/370). Lithium-induced nuclear translocation of b-catenin was also significantly reduced in the PS2 Met239Val mutant fibroblasts (285/456). Nuclear translocation in the group of mutant PS1 fibroblasts was more affected than in mutant PS2 fibroblasts. This observation is in accordance with the reduced clinical penetrance and the later age of disease onset in families with PS2 mutations (mean ~ 65 years versus ~45 years for PS1 mutations) as described in *Bird et al.*, 1997, *Ann. Neurol.* 40:932-936. Similar results were obtained with the HEK293 cells. Thus, nuclear β-catenin levels substantially increased in HEK293 cells expressing either endogenous PS1/PS2 (6.8) fold),

transfected wild type human PS1 (6.9) fold), or wild type PS2 (6.9) fold). In contrast, nuclear β-catenin levels increased only 2.1 fold in HEK293 cells with mutant PS1 (PS1 Leu392Val: 4.4 fold; PS1 Δ290-319: 3.5 fold) and only 1.2 fold in cells expressing mutant PS2 (PS2 Asn141Ile: 1.8 fold). These differences in nuclear β-catenin are not associated with differences in total cellular β-catenin, and cannot be ascribed to differences in PS1 levels, because despite the fact that untransfected HEK293 cells express far lower levels of endogenous PS1, nuclear β-catenin levels were higher in lithium treated untransfected HEK293 cells than in the mutant PS1 transfected HEK293 cells. Finally, in contrast to the changes in nuclear translocation of β-catenin, nuclear translocation of NFκB in response to Tumour Necrosis Factor-α was not affected by PS1 mutations. The latter observation argues that alterations in β-catenin mediated signal transduction mechanisms associated with presenilin mutations does not arise from a non-specific abnormality in nuclear protein transport.

To confirm that the effect of PS1 missense mutations was a specific effect of pathogenic amino acid substitutions and did not occur with non-pathogenic substitutions, these experiments were repeated in fibroblasts from a heterozygous carrier of the PS1 Glu318Gly polymorphism (which is not associated with increased risk for AD or with abnormal bAPP processing - (*Mattila et al.*, 1998, *Ann. Neurol.* in press. Both basal nuclear b-catenin levels (29/489 nuclei) and Li^+ induced nuclear translocation (343/451 nuclei) in the PS1 Glu318Gly fibroblasts were indistinguishable from normal control fibroblasts.

Based on these discoveries, Applicants believe that some of the PS1 mutations in the large cytoplasmic loop might directly disrupt the putative PS1:*arm* interaction domain at residues 372-399. However, quantitative liquid β-galactosidase assays indicate that there is not a large difference between the yeast-two-hybrid interaction of hNPRAP with wild type $PS1_{266-409}$ loop bait sequences compared to its interaction with the mutant Leu286Val $PS1_{266-409}$ bait sequences (βgal activities ± SEM: Wild-type = 7.99 ± 0.33; L286V = 6.90 ± 0.50 units, p =

n.s.). Furthermore, several of the mutations that were tested affect residues remote from the cytoplasmic loop. An alternate explanation revolves around a putative role for the presenilins and homologous proteins such as SPE4 in the docking and trafficking of a subset of cellular proteins such as the major sperm protein in the case of SPE4. In this regard, it may be relevant that another class of *armadillo* containing proteins, the importins (*Gorlich*, 1997, *Curr. Opin. Cell Biol.*, 9:412-419), are involved in the facilitation of translocation of proteins across the nuclear membrane (which together with the endoplasmic reticulum is a major intracellular site of presenilin protein expression (*De Strooper et al.*, 1997, *J. Biol. Chem* 272:3590-3598; *Walter et al.*, 1996, *Molec. Medicine* 2:673-691)). Presenilin mutations may cause a dominant gain of aberrant function by causing anomalous and/or misdirected trafficking of a limited number of interacting partners. This would be in agreement with results which suggest that presenilin mutations are associated with mistrafficking of βAPP (*Martin et al.*, 1995, *NeuroReport* 7:217-220; *Scheuner et al.*, 1996, *Nature Med.*, 2:864-870; *Citron et al.*, 1997, *Nature Med.* 3:67-72; *Duff et al.*, 1996, Nature 383:710-713; *Borchelt et al.*, 1996, *Neuron*, 17:1005-1013).

Mutations in PS1 and PS2 may cause abnormalities in transcriptional activity in response to receptor mediated signals. Alternatively, by disturbing the intracellular compartmental distribution of *arm* proteins such as β-catenin, mutations in PS1 or PS2 may disturb their function at inter-cellular junctions. Interactions between catenins (such as β-catenin or APC) and cadherins (such as N-cadherin or cadherin-14) are thought to be important in the maintenance of CNS synapses (*Bhat et al.*, 1994, *J. Neurosci.* 14:3059-3071; *Takeichi*, 1995, *Curr. Opin. Cell Biol.* 7:619-627; *Uchida et al.*, 1996, *J. Cell Biol.* 135:767-779; *Shibata et al.*, 1997, *J. Biol. Chem.* 272:5236-5240). Synaptic dysmorphism and synaptic loss is a prominent part of the pathology of AD (*Masliah et al.*, 1993, *Brain Path* 3:77-85; *Jellinger*, 1996, *J. Neural Trans.* 47 (Suppl.):1-29).

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

All patent and non-patent publications cited in this specification are indicative of the level of skill in the art to which this invention pertains. All these publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.